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Localization and mRNA expression of CY3A and P-gp in human duodenum as a function of age

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Running title: postnatal expression of CYP3A and P-gp in human duodenum

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ABSTRACT

Cytochromes P450 3A (CYP3A) and P-glycoprotein (P-gp) are mainly located in enterocytes and hepatocytes. The CYP3A/P-gp system contributes to the first pass metabolism of many drugs, resulting in a limited bioavailability. During the neonatal period, a shift between CYP3A7, the fetal form, and CYP3A4 occurs in the liver but data on the expression of the CYP3A/P-gp complex in the intestine are very limited. 59 normal duodenal biopsies from Caucasian children aged 1 month to 17 years were studied. Localization of the proteins by immunohistochemistry analysis was performed using a polyclonal antibody “Nuage” anti-CYP3A and a monoclonal antibody “C494” anti-P-gp. The mRNA quantification was performed using highly specific real time RT-PCR. Villin mRNA quantification was used for normalization. CYP3A protein was detected in all enterocytes in the samples from patients over 6 months of age while it was not in younger samples. P-gp protein was expressed at the apical and upper lateral surfaces of the enterocytes. CYP3A isoforms and P-gp mRNA levels were highly variable. CYP3A4 and CYP3A5 mRNA levels were high during the first year of life and decreased with age while CYP3A7 was detected at a low level in 64% of samples, whatever the age. P-gp mRNA expression level was also highly variable. Our results showed that neonates and infants had a significant expression of CYP3A and P-gp mRNA in the intestine, suggesting a different maturation profile of CYP3A and P-gp with age in the liver and the intestine.

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INTRODUCTION

Cytochrome P450 isoenzymes are part of the Phase I metabolizing enzymes. The CYP3A subfamily is predominant accounting for almost 30% of the cytochromes in the liver and 70% of the cytochromes in the small intestine and involved in the metabolism of more that 70% of currently administered drugs (Paine et al., 1997; Wacher et al., 1998). The CYP3A subfamily consists of three major isoforms: CYP3A4, CYP3A5, CYP3A7 and a recently identified CYP3A43. They are closely related as they share at least 83% amino acid sequence identity. In adults, CYP3A4 is the predominant form in the liver and the intestinal tract (Koch et al., 2002). It is variably expressed but none of the identified genetic polymorphisms showed relevant clinical effect (Lown et al., 1994; Wacher et al., 1998; Wandel et al., 2000). CYP3A5 is present in the liver (Schuetz et al., 1994; Wrighton et al., 1990) and intestine (Koch et al., 2002). The variability of CYP3A5 expression is under genetic control. The contribution of CYP3A5 to drug metabolism is important as CYP3A5 can contribute to over 50% of total CYP3A content (Hustert et al., 2001; Kuehl et al., 2001). CYP3A7 is the major CYP isoform detected in embryonic, fetal and newborn liver (Schuetz et al., 1994; Lacroix et al., 1997). It is also expressed, at a low level, in the adult liver and intestine (Koch et al., 2002). P-glycoprotein (MDR1) is a plasma membrane glycoprotein belonging to the ATP-binding cassette transporters superfamily (Tanigawara, 2000) acting as an efflux system contributing to the protection of the organism. It is located in many tissues and specifically within the brush border at the apical surface of mature enterocytes in the small intestine (Thibeaut et al., 1987) Its expression is genetically controlled (Brinkmann et al., 2001; Cascorbi et al., 2001). CYP3A and P-gp share common substrates and their coordinated action in the intestine results in a reduced bioavailability of drugs administered orally (Wacher et al., 1998; Paine et al., 1996). Although many of these drugs are used in children, studies concerning the intestinal ontogeny of CYP3A and P-gp are scarce. In the present study, we report

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new data regarding the localization and the mRNA expression of the three isoforms of CYP3A and P-gp in the duodenum of infants and children aged one month to 17 years.

MATERIALS AND METHODS

Patient samples

Fifty nine duodenal biopsies (second or third part of duodenum) from Caucasian patients aged 1 month to 17 years, included in the tissue bank of the department of Anatomopathology, were selected retrospectively. These biopsies were performed for medical investigations (growth retardation, gastrointestinal reflux or pain, hypotrophy or diarrhea) and were found normal after histological examination. Biopsies from patients receiving known inducers or inhibitors of CYP3A and P-gp were excluded. The study was conducted according to the French legislation. Snap frozen tissues were used for mRNA quantification and formalin embedded tissues were used for immunohistochemistry analysis.

Immunohistochemistry analysis

Duodenal biopsies' staining was performed using a 3-step immunoperoxidase (sandwich) technique. For CYP3A, the primary antibody was a rabbit anti-human polyclonal CYP3A antibody called «Nuage»; the secondary antibody was a goat anti-rabbit antibody purchased from ICN Biochemical (Cleveland, Ohio) and the tertiary antibody was a rabbit peroxidase anti-peroxidase complex from Dako (Carpinteria, CA). For P-gp, a mouse anti-human monoclonal P-gp antibody called «C494» was purchased from Dako, the biotinylated sheep anti-mouse antibody from Silenus / Chemicon (Temecula, CA) and the streptavidine peroxidase complex from Boehringer (Ingelheim, Germany) The detection for both reactions was conducted with DiAmino-Benzidine (DAB KIT from Menarini: Italy). Negative controls (without adding the primary antibody) showed absence of staining and adult liver and intestine sections were used for

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the validation of the CYP3A and P-gp specific staining (data not shown). Sections were viewed using a microscope (Leitz-Laborlux. Germany).

RT-PCR

Extraction of total RNA and Reverse transcription

Total RNA was extracted from human duodenal biopsies according to the method described by the manufacturer with the RNA Plus solution (QBIOgene: Illkirch, France). The reverse transcription (RT) reaction was performed on 1 µg of the extracted total RNA.

Quantitative real time PCR (QRT-PCR) with TaqMan detection

The QRT-PCR analysis system allows an accurate, specific and reproducible quantification of mRNA. Forward and reverse primers and the fluorogenic TaqMan probe for CYP3A isoforms and villin were designed to span exon junctions in the fully processed mRNA using Primer Express software (Applied Biosystems: Foster City, CA). P-gp primers and probe sequences were the courtesy of Dr Barbu. FAM was used as the 5'-fluorescent reporter while TAMRA was added to the 3' end as a quencher for CYP3A5, CYP3A7, P-gp and villin probes and an MGB-probe was used for CYP3A4. Primers and probes sequences are listed in table1. Primers were purchased from Genset / Proligo (Colorado, USA) and probes from Applied Biosystems.

PCR conditions

50 ng of cDNA resulting from RT were subjected to PCR amplification in the ABI 7700 Sequence Detection System (Applied Biosystems). For CYP3A5, CYP3A7, P-gp and villin, the final reaction conditions were: 200 nM of forward and reverse primers, 100 nM of the probe and 1X Master Mix (Applied Biosystems). For CYP3A4 we used the Core Reagent Mix (Applied Biosystems) with 400 nM of primers, 100 nM of MGB probe and 7 mM $MgCl_2$. For 18S, we used the Applied Biosystems kit (ref 4319413E). PCR conditions for the 6 genes were: 2 min at

50°C, 10 min at 95°C followed by 50 cycles each of 15 s at 95°C and 1 min at 60°C. Each sample was analyzed in duplicate. Negative and positive controls were present in all the assays. Data were analyzed using the Sequence Detector V1.6 program (Applied Biosystems).

PCR specificity control and normalization

The specificity of the assays was determined using plasmids specifically expressing the gene of interest. The two plasmids expressing, respectively, CYP3A4 and CYP3A5 were the courtesy of Pr P.Beaune, the one expressing CYP3A7 the courtesy of Dr T.Cresteil and the one expressing P-gp the courtesy of Dr V.Barbu. The villin plasmid was constructed using the TOPO TA cloning® kit with pCR®2.1-TOPO as the plasmid vector (Invitrogen: Carlsbad, CA). The assay specificity was verified for each CYP3A isoform by testing the amplification of primers and probes of the gene of interest versus the plasmids of the 2 other isoforms. In all cases, the amplification with the target gene was at least 10^5 fold higher than with the other isoforms. CYP3A5's probe recognizes the corresponding pseudogene. Standard curves were constructed with the use of serial 10-fold dilutions ranging from 10^2 to 10^7 copies of the plasmid containing the target gene. Villin gene expression was chosen to normalize the different mRNA quantifications with respect to the depth of the biopsy and proteolysis. The ubiquitously expressed gene 18S, was used to control the extraction and the RT step efficacy. Data used for statistical tests were expressed as the ratio of the number of copies of the target gene mRNA over villin mRNA copies number (ratio of the target gene over V. ex: CYP3A4 / V).

STATISTICS

Statistical analyses were conducted with the use of STATA Software version 7 (STATA Corporation). Quantitative variables by age subgroups were expressed as percentiles because of

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skewed distribution. The relationships between age and the different genes were assessed using median regression. A p-value less than 0.05 was considered statistically significant.

RESULTS

Immunohistochemical localization of CYP3A and P-gp proteins

CYP3A protein was detected in all biopsies and located within enterocytes. In the samples obtained from patients younger than 6 months of age, CYP3A protein was detected in half of the enterocytes (staining in cells with no staining in adjacent ones) (fig 1A) while it was expressed in all the cells in older patients (fig 1B). P-gp protein was detected in all the enterocytes and located on the apical surface (fig 1C/D). In children aged less than 3 years, additional staining was located on a limited upper part of the lateral surface. The expression variability of both CYP3A and P-gp proteins was observed between enterocytes in the same villous tip and between adjacent villi.

CYP3A and P-gp mRNA quantification

A significant correlation was evidenced between 18S and villin mRNA ($n=59$, $r=0.92$; $p<0.001$). The mRNAs of the three different CYP3A isoenzymes were quantified separately and their expressions were highly variable between samples. CYP3A4 and CYP3A5 mRNA levels were in the same order of magnitude while CYP3A7 levels were much lower. P-gp mRNA levels were similar to those of CYP3A4 although P-gp was overexpressed in some samples. For CYP3A4, the mRNA quantification varied more than 100 fold with a 3A4 / V mRNA ratio ranging from 4×10^{-4} to 0.5. There was a significant relation between CYP3A4 and age ($p=0.0001$). Expression was high in the first year of life (CYP3A4 / V ratio from 0.04 to 0.5) and decreased with age to reach lower values in older children (CYP3A4 / V ratio from 0.01 to 0.2). CYP3A5 mRNA was

expressed in all but one sample. CYP3A5 / V ratio ranged from 10^{-3} to 0.46 and decreased with age without reaching statistical significance (p=0.061). CYP3A7 mRNA was evidenced in 37/58 (64%) patients but at a much lower level than CYP3A4 and CYP3A5. CYP3A7 / V ratio ranged from 16×10^{-6} to 5×10^{-4} with two patients showing relatively high ratios of 0.001 and 0.002 but with no statistical relation with age (p=0.97). Pooled CYP3A mRNA, expressed as the CYP3A / V ratio was higher in the first year of life (CYP3A / V ratio ranging from 0.029 to 0.9) than in older children. CYP3A4 mRNA accounted for 58.6 ± 20% of total CYP3A mRNA expression (range: 17.5 to 100%). CYPA5 contributed to more than 50% of total CYP3A mRNA when CYP3A5 / V ratio was higher than 0.08. P-gp mRNA was detected in all the samples. Here again, expression was highly variable between samples, P-gp / V ratio ranging from 0.005 to 4 with 9 patients having a ratio higher than 1.3. No significant relation was found between P-gp and age (p=0.195).

In order to get insight into the impact of age on the CYP3A and P-gp mRNA expression, our population was arbitrary divided into 3 groups: group 1: patients aged 1 month-1 year (n=19), group 2: 1-6 years (n=16) and group 3 patients over 6 years of age (n=24). 18S and villin mRNA levels were not different between the three groups (data not shown). The high interindividual variability in the expression found for the CYP3A isoforms as well as for P-gp was highlighted by using percentiles as shown in fig 2. The dispersion was higher in the group 1 (1 month-1 year) when compared to groups 2 and 3 for CYP3A4 and CYP3A5.

DISCUSSION

CYP3A and P-gp are present in many tissues, but primarily in the liver and gut. However, very few studies investigated the postnatal development of the CYP3A/P-gp system in humans, as

tissues from neonates, infants and children are difficult to collect. In the present study, the duodenal biopsies were taken for medical investigations. The very small amount of tissue did not allow to determine specific protein content or activity but immunohistochemical localization and mRNA quantification were performed.

For CYP3A, we observed a staining heterogeneity between adjacent enterocytes of the same villus in patients aged less than 6 months while the protein was uniformly expressed in older biopsies. A heterogeneous expression of CYP3A protein had been previously reported in hepatic biopsies from children older than 6 months and adults (Ratanasavanh et al., 1991). These results suggest that the developmental profile of CYP3A might be cell dependent. The P-gp protein localization was reported to be apical but not lateral in the rare available data obtained in adults using “MRK16” antibody (Thibeaut et al., 1987). As an experimental artifact was ruled out by testing “C494” antibody on adult liver and intestine biopsies, this new undescribed lateral P-gp localization reported in our study might be the result of developmental process.

In order to quantify the mRNA, we developed a very specific and sensitive TaqMan method that showed no cross-amplification between the 3 CYP3A isoforms. Our mRNA quantification results, normalized to villin, were in the same order of magnitude as those reported by Nishimura et al (Nishimura et al., 2003) on a pooled small intestinal total RNA.

The CYP3A protein content in the intestine was reported to gradually increase in the first months of life in the literature (Johnson et al., 2001) which is not consistent with the mRNA level decrease reported in our study. This dissociation between mRNA and protein contents during the maturation process was already reported for CYP2D6 (Treluyer et al., 1991). This might reflect a posttranscriptional regulatory mechanism, with the possible implication of a local intestinal trigger factor, in the activation of translation. We were able to detect CYP3A7 mRNA in 64% of our duodenal samples. Its level of expression was much lower than CYP3A4 and did not vary

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with age. According to our data, the contribution of CYP3A7 in the overall CYP3A dependent enterocytic metabolism is very limited in children. The interindividual variability in CYP3A5 mRNA expression was important with high expression in a limited number of samples. The pharmacogenetically dependent expression of CYP3A5 was not investigated in the present study, although the high levels of CYP3A5 protein expression were previously associated with a genetic polymorphism in intron 3 of CYP3A5 (Hustert et al., 2001; Kuehl et al., 2001). Many studies described the hepatic ontogeny of CYP3A isoforms. CYP3A7, detected in fetal liver was replaced by an increasing protein and activity levels of CYP3A4 after birth (Lacroix et al., 1997; Kearns et al., 2003). In contrast, in our duodenal samples, after birth, CYP3A4 mRNA expression decreased significantly after the first month of life to reach adult levels. This discrepancy in the expression profile of the 3 isoforms between the liver and the intestine was already observed in rats (Johnson et al., 2000) and suggest that an organ dependant factor might control the expression of the different isoenzymes. Additional studies are required to confirm these findings and to obtain additional data from other duodenal / jejunal segments of the intestine.

In contrast to CYP3A isoforms expressions, the ontogenic profile of P-gp mRNA and protein content are in agreement. In the literature, in humans, P-gp mRNA and protein were detected as early as 11 to 14 week's gestation in the liver and the kidney but only detected in the intestine in older samples (van Kalken et al., 1992). Similarly, P-gp mRNA was present in all our samples, from 1 month until adulthood with a large interindividual variability.

The dissociation between the CYP3A mRNA level reported in the present study and protein content and activity found in the literature highlights the possibility of a post-transcriptional regulation which should be supported by additional researches. In addition, in the intestine, food and orally administered xenobiotics might trigger CYP3A4 and CYP3A5 protein synthesis in the first months of life, playing a protective role against xenobiotic exposure. Additional studies

would allow to determine if the local ontogeny of drug metabolizing enzymes and transporters in the intestine might depend on the type of diet and the time of diet diversification.

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FOOTNOTES

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LEGENDS FOR FIGURES

Figure 1. Immunohistochemistry on formalin embedded duodenal tissue sections with anti-CYP3A polyclonal antibody “Nuage” and anti-P-gp monoclonal antibody “C494”. Some enterocytes do not express CYP3A in the samples from patients aged less than 6 months while all enterocytes express CYP3A protein in biopsies from older patients. A: arrow showing enterocyte not expressing CYP3A (biopsy from a 48 day old patient). B: all enterocytes express CYP3A (biopsy from a 10 year old patient). P-gp is located on the apical and lateral surfaces of the enterocytes. C: apical staining of P-gp (biopsy from a 7 year old patient). D: lateral staining of P-gp (biopsy from a 3 month old patient).

Figure 2. mRNA copies / villin mRNA copies ratio box-plots and percentiles tables for A: CYP3A4, B: CYP3A5, C: CYP3A7 and D: P-gp.

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Table 1. Primers and probes sequences for real time quantitative PCR (TaqMan).

CYP3A4	Forward primer (631-657): 5'-AAGAAGCTTTTAAGATTTGATTTTTTG-3'
(GenBank accession	Reverse primer (730-703): 5'-ACACAGATATTTAATACTTCAAGAAT-3'
number AF182273)	Probe (677-698): 5'-TAACAGTCTTTCCATTCCTCAT-3' (MGB)
CYP3A5	Forward primer (258-279): 5'-CTGGAAATTTGACACAGAGTGC-3'
(GenBank accession	Reverse primer (403-377): 5'-TTCGATTTGTGAAGACAGAATAACATT-3'
number NM_000777)	Probe (306-334): 5'-AACGTATGAAGGTCAACTCCCTGTGCTGG-3'
CYP3A7	Forward primer (636-661): 5'-AAGATTTAATCCATTAGATCCATTCG-3'
(GenBank accession	Reverse primer (782-754): 5'-CGACCTTCTTTTATCTGTTTTACAGATTT-3'
number NM_000765)	Probe (674-707): 5'-AAGTCTTTCCATTCCTTACCCCAATTCTTGAAGC-3'
P-glycoprotein (P-gp)	Forward primer (3103-3123): 5'-TGGGAAGATCGCTACTGAAGC-3'
(GenBank accession	Reverse primer (3226-3201): 5'-TTTCCTCAAAGAGTTTCTGTATGGTA-3'
number M14758)	Probe (3131-3161): 5'-AACTTCCGAACCGTTGTTTCTTTGACTCAGG-3'.
Villin	Forward primer (1042-1061): 5'-GCCGTCTTTCAGCAGCTCTT-3'.
(GenBank accession	Reverse primer (1143-1124): 5'-CACCTGTTCCACTTTGGCCA-3'
number XM_010866)	Probe (1063-1086): 5'-CAGAAGTGGACAGCGTCCAACCGG-3'.

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Figure 1

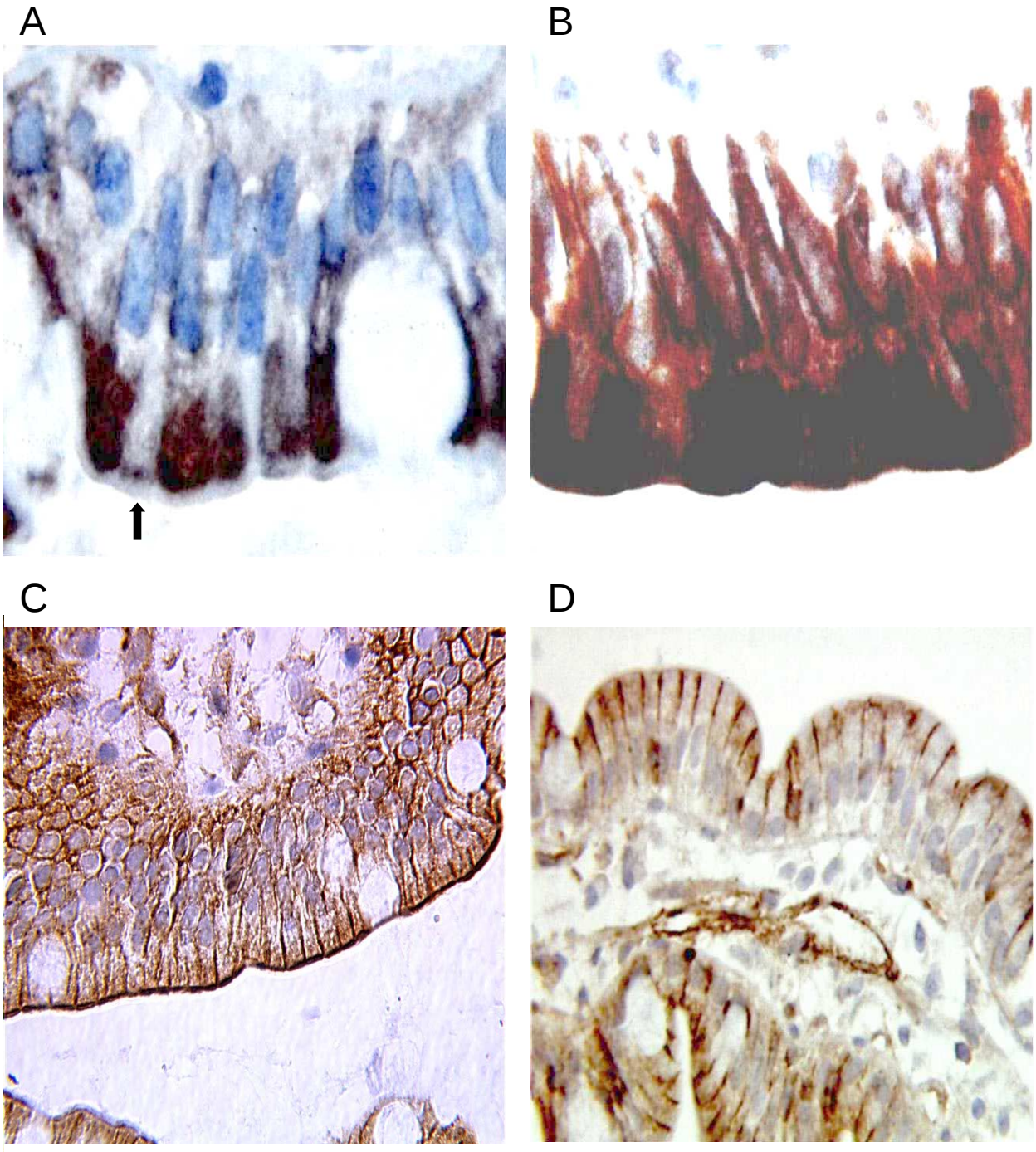

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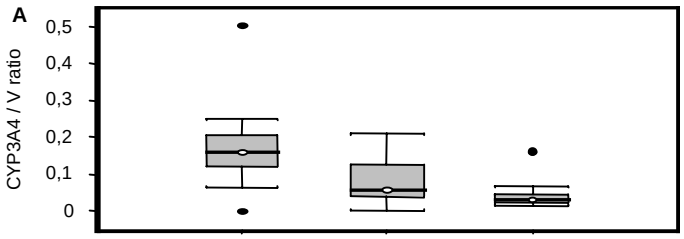
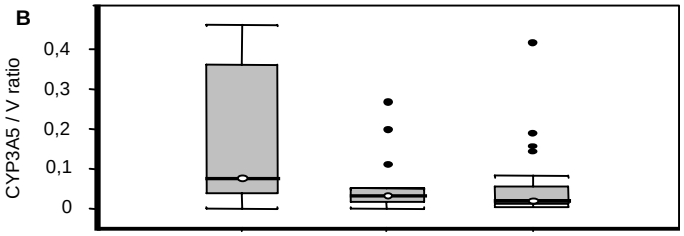
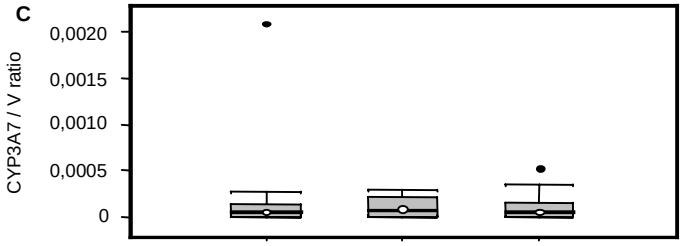
Figure 2



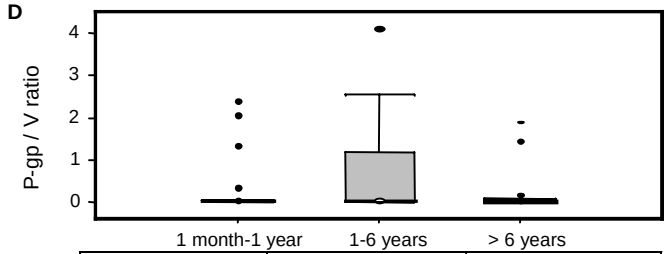
1 month - 1 year					1 year - 6 years					> 6 years				
5 th	25 th	50 th	75 th	95 th	5 th	25 th	50 th	75 th	95 th	5 th	25 th	50 th	75 th	95 th
0	0,1	0,16	0,21	0,5	0	0	0,06	0,13	0,21	0	0	0,03	0,05	0,16



1 month - 1 year					1 year - 6 years					> 6years				
5 th	25 th	50 th	75 th	95 th	5 th	25 th	50 th	75 th	95 th	5 th	25 th	50 th	75 th	95 th
0	0	0,08	0,36	0,5	0	0	0,03	0,05	0,27	0	0	0,02	0,05	0,19



1 month - 1 year					1 year - 6 years					> 6 years				
5 th	25 th	50 th	75 th	95 th	5 th	25 th	50 th	75 th	95 th	5 th	25 th	50 th	75 th	95 th
0	0	6E-05	1E-04	2E-03	0	0	9E-05	2E-04	3E-04	0	0	5E-05	1E-04	3E-04



1 month - 1 year					1 year - 6 years					> 6 years				
5 th	25 th	50 th	75 th	95 th	5 th	25 th	50 th	75 th	95 th	5 th	25 th	50 th	75 th	95 th
0	0	0,03	0,05	2,4	0	0	0,04	1,18	4,1	0	0	0,02	0,07	1,42